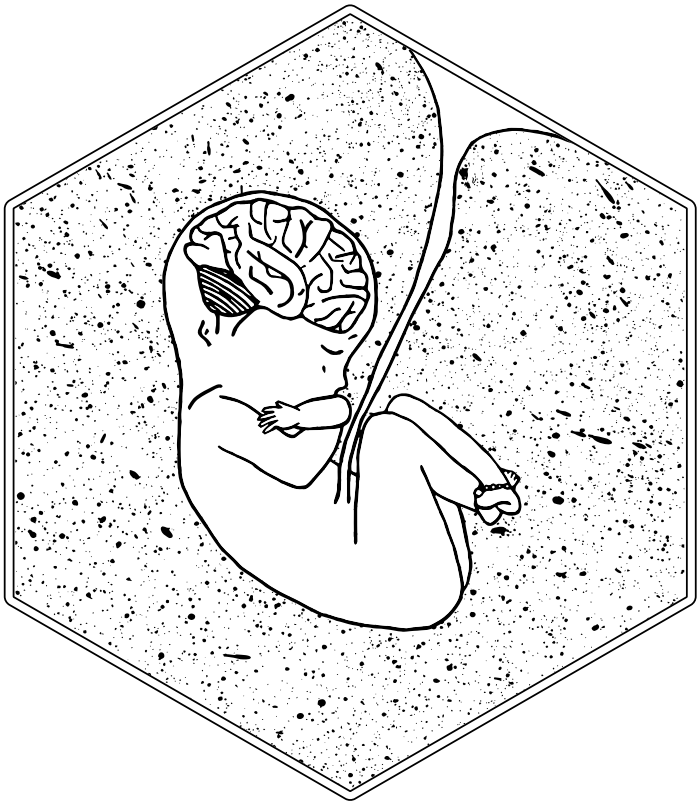


INTRODUCTION

INTRODUCTION

Our brain is a universe of neurons. All these neurons interact and together result in something that seems bigger than the sum of its parts. Our brain affects the way we perceive the world, how we react to others, determines our personality and shapes our behaviour. In our society, one of the most salient characteristics that determines how we see each other and how we value behaviour is the sex of an individual. Although there are actually a lot of similarities between behaviour of men and women, there are indeed also certain differences. Despite still being heavily debated, most of these sex differences are probably a consequence of sexual differentiation of the brain under the influence of environmental and biological factors.

There are two major events during life when dramatic changes in the body and the brain occur. Early in development, the body and the brain are first undifferentiated and then follow their own developmental course in male or female direction. Later in life, during puberty, the body becomes sexually reproductive and our brain is adapted to the new phase of life. This sexual differentiation of the body and brain during life is the result of a continuous interplay between genes, sex hormones, and the external environment.

Genes contain crucial information for the developmental process, but information from the external environment is indispensable for their expression and their translation from genotype to phenotype. This is where hormones come into play. The word 'hormone' comes from the Greek word "ὁρμῶ", which means "to set in motion". The brain receives information about the internal and external environment and translates this into hormone production. These hormones induce via mRNA transcription a physiological response. This way, the body and brain are adapted to the environment and phase of life. So, the brain has a dual role with regard to hormones, because it both controls the hormone production and is subject to the influence of hormones itself.

Sex hormones can affect the structural and functional development of the brain, and subsequently have essential influences on the way we perceive and react to the world around us. This makes the influence of hormones on the brain extremely interesting to study.

Central in this thesis is the role of sex hormones on the sexual differentiation of the brain and behaviour at several stages during development. This broad topic is investigated by studying two fundamental research questions that have revealed sex differences. At the brain level the division of functions between both hemispheres is investigated, and at the behavioural level the development of gender-typical behaviour.

SEX HORMONES

Let's first clarify some terminology. Sex hormones belong to the class of steroid hormones, which

are mainly produced by the gonads. Therefore, they are also known as gonadal hormones. Sex hormones encompass different sub-classes of hormones: androgens, estrogens and progesterone. Androgens are also referred to as male hormones, because the levels are generally higher in men than in women. On the other hand, estrogens are known as female hormones, because the levels of these hormones are higher in females. However, men and women *both* produce androgens and estrogens.

TESTOSTERONE has received most attention in studies on the sexual differentiation of the brain in humans, and has a central role in all chapters of this thesis.

ESTRADIOL is the most well-known estrogen. Testosterone and estradiol have a rather similar molecular structure. Therefore, testosterone can easily be converted into estradiol with help of the enzyme aromatase (see Figure 1). In contrast to what is known in other mammal species, it is thought that testosterone directly masculinizes the brain in humans, but it is possible that estradiol has a role in masculinizing or feminizing the human brain as well (Arnold and McCarthy, 2016; McCarthy, 2008; Peper et al., 2011b).

PROGESTERONE is one of the important precursors for the production of androgens, and might also have effects on the sexual differentiation of brain and behaviour (Peper et al., 2011b).

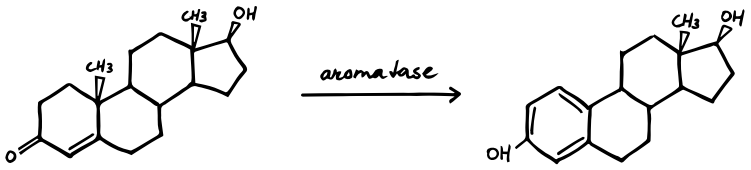


Figure 1 *Conversion of testosterone to estradiol by aromatase.*

A SMALL RECAP ON SEXUAL DIFFERENTIATION OF THE BODY

The first 7 weeks after conception, human embryos are mostly sexual undifferentiated. But then, based on the presence of the SRY-gene on the Y-chromosome, testes will develop in male embryos. Around 9 weeks of gestation, the testes will start producing androgens, which results in a huge surge of testosterone (see Figure 2). In boys, the production of androgens by the now developed testes will subsequently result in the development of male sex organs and genitalia. In the first 3 months after birth, the "mini-puberty", there is also a testosterone peak in boys which further masculinizes the body and brain (Kurtoğlu and Baştuğ, 2014). The next peak occurs in boys in puberty, when there is a surge of testosterone causing the development of secondary sex characteristics.

In girls, female sex organs and genitalia develop in the absence of high androgen levels early in gestation. There are 3 peaks in estradiol production in girls, parallel to the testosterone peaks in boys (Figure 2). However, the function of the estradiol peaks before birth and in mini-puberty is

elusive (Arnold and McCarthy, 2016; Kurtoğlu and Baştuğ, 2014). In puberty, elevated estradiol levels cause the development of secondary sex characteristics.

The most recent theory on the sexual differentiation of the body and brain states that sexual differentiation is always a result of a multifactorial process, including effects of sex chromosomes and sex hormones like testosterone and estradiol, acting together or in parallel, and reacting to environmental factors (Arnold, 2017).

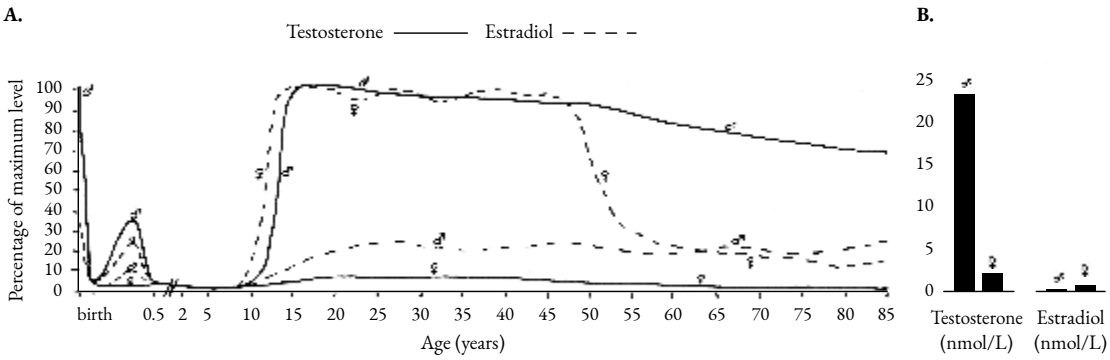


Figure 2 *A. Testosterone and estradiol levels in men and women during lifetime. Levels are shown as the percentage of the maximum level across sexes. The figure is adapted from Ober et al. (2008). B. The absolute mean maximum testosterone and estradiol level in men and women in adulthood (Khosla et al., 1998). Men have higher testosterone levels than women, and women higher estradiol levels than men. In both sexes testosterone levels are higher than estradiol levels, but estradiol is effective at a lower concentration.*

SEXUAL DIFFERENTIATION OF THE BRAIN

The development of the brain is a remarkable process. Cells are born, differentiate into neurons, and migrate to their final destination. The neurons form dendrites and axons, who subsequently form synaptic connections with other neurons, together forming a complex network called "the nervous system". After about 100 days of gestation the brain is already recognizable in its mature form, but the brain continuously develops and is plastic.

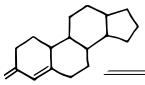
Hormones act on the brain via sex hormone receptors. From the moment the brain starts to develop, cells in the entire nervous system already have these receptors (Arnold and McCarthy, 2016), so the brain is directly influenced by sex hormones (Swaab, 2007). Simply put, sex hormones can differentiate the brain anatomy in 2 ways: by cell birth or cell death. Via this mechanism, the brain anatomy is dynamic during life: neurons grow and die, dendrites and axons grow and retract, and synapses come and go. Interestingly, one would expect that sex differences mostly derive from the growth of new cells; however, cell death seems to be the driving force behind the sexual

differentiation. For example, in puberty the thinning of the frontal cortices is more accelerated in girls than in boys as testosterone levels increase (Bramen et al., 2012). In most brain regions, neurons are overproduced and eliminated by as much as 50%, especially before birth and during puberty (Andersen, 2003).

IMPORTANT PERIODS: BEFORE BIRTH AND IN PUBERTY

The classic theory on sexual differentiation of the brain introduced two influential terms: organizing and activating effects of sex hormones (Phoenix, 1959). Organizing effects are structural and permanent effects on the brain that generally occur during a sensitive period. Historically, the prenatal and neonatal periods were seen as the sensitive periods for organizing effects. Activating effects occur on top of or based on organizing effects, are temporary and may occur during the entire lifespan. However, already in 1985 Arnold and Breedlove reported that the distinction between the two is not absolute. Recently it became established that puberty is another period in which hormones can have organizing effects on the brain (Blakemore et al., 2010; Peper and Dahl, 2013; Romeo, 2003; Sisk and Zehr, 2005), resulting in sex differences (Blakemore et al., 2010; Peper et al., 2011a, 2009; Perrin et al., 2008; Raznahan et al., 2010).

In this thesis I specifically focus on the prenatal and pubertal periods, when testosterone and estradiol levels rise to their peak levels and major developmental changes occur in the brain.



SEXUAL DIFFERENTIATION OF BRAIN LATERALIZATION

The sexual differentiation of the brain can be studied at many levels, from sex differences in cell size to sex differences in brain size, and from anatomy to functional brain activation. In this thesis, I investigate sex differences in the brain at a rather global level: the difference in activation between the left and the right hemisphere during cognitive tasks, called brain lateralization.

Both hemispheres differ in how, and to what extent, they control motor skills, perception and cognition. For example, in most people the left hemisphere is more involved in language than the right hemisphere, while the right hemisphere is more involved in visuospatial orientation than the left hemisphere. Thus, the majority of the general population shows a similar direction in the pattern of lateralization of functions, but there is also considerable individual variation (Hirnstein et al., 2008, Lust et al., 2011b).

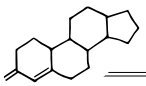
There are small but consistent differences in lateralization between the sexes (for a review see Lust et al., 2010). The most apparent is the finding that more men than women are left-handed or ambidexter (Papadatou-Pastou et al., 2008), and men are also found to be stronger lateralized than women on language and spatial orientation tasks (Beltz et al., 2013). This inspired the hypotheses that exposure to sex hormones may organize the development of brain lateralization during a sensitive prenatal period. It has been suggested that the development of lateralization is part of the normal process of sexual differentiation (Hines & Shipley, 1984). Another much cited hypothesis claims

that prenatal testosterone affects both hemispheres differentially, as both hemispheres differ in the timing of development (Geschwind and Galaburda, 1985). A few years later, evidence accumulated that prenatal exposure to testosterone induces pruning of the corpus callosum, which is the main connection between the left and right hemisphere. This would reduce crosstalk between the hemispheres, thereby potentially increasing the strength of lateralization (Witelson & Nowakowski ,1991). Note that these hypotheses only assume an influence of prenatal testosterone, because they were formulated in a time when it was not recognized that hormones later in life, specifically in puberty, could also have organizing effects on the brain. This may have contributed to the fact that 32 years after the first theory of sexual differentiation of brain lateralization was formulated, the influence of sex hormones on lateralization is still elusive.

For a long time, brain lateralization was thought to be a trait that only humans possessed, but more recently it was discovered that brain lateralization is present in all vertebrates and at least some invertebrates. Several scientists therefore argue that brain lateralization was already present when the earliest vertebrates evolved 500 million years ago (MacNeilage et al., 2009; Meguerditchian et al., 2010; Rogers et al., 2004). The fact that brain lateralization is present is so many species suggests a fundamental principle in the organization of brain and behaviour and thereby that evolution has positively selected on some benefit of having a lateralized brain. The discovery that also non-human animals have a lateralized brain gave an impulse to experimental studies investigating the effects of sex hormones on behavioural and brain lateralization. However, there is still a gap between non-human animal and human studies. In animals it is difficult to investigate functional brain lateralization and cognition, while in humans it is difficult to obtain prenatal hormone data and experimental manipulation of hormones is not acceptable for the obvious ethical reasons.

In this thesis the role of sex hormones on the sexual differentiation of the brain and behaviour is investigated by studying two fundamental research questions. The first research question is:

What is the influence of prenatal and pubertal sex hormones on brain lateralization?



SEXUAL DIFFERENTIATION OF GENDER DEVELOPMENT

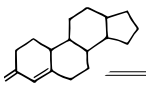
In the last part of the thesis I will shift from the effects of sex hormones on the brain to effects on behaviour. The focus is on behaviour that typically differs between boys and girls. Sex differences in behaviour and interests emerge in early childhood and are clearly observable and measurable in play behaviour and toy preferences (O'Brien & Huston, 1985; Campbell et al., 2000). Thus far, three studies have investigated the relationship between prenatal sex hormones measured in

amniotic fluid and play behaviour at different ages (13 months: Van de Beek et al., 2009a; 5 years: Knickmeyer et al., 2005; and 6-10 years: Auyeung et al., 2009). However, these studies produced mixed outcomes, possibly because different ages were investigated with different methods. There is need for a longitudinal study investigating the effects of prenatal sex hormones on play behaviour at multiple ages using the same methodology. Moreover, it is interesting to investigate whether childhood play behaviour predicts subsequent gender development in puberty.

In adolescence, there are clear sex differences in gender identity (the *sense of self*), gender role (the *behaviour*) and sexual orientation. These traits go through a transitional phase in puberty (Hines, 2011), possibly under the influence of sex hormones rising to high levels at this time. However, there is – to the best of my knowledge – no literature investigating the relationship between pubertal sex hormones and gender development in the normal population. Interestingly, in an "atypical population" of children diagnosed with Gender Dysphoria, only 15% of these children fulfill the criteria for Gender Dysphoria (DSM-5, APA 2013) later in adolescence (Steensma et al., 2013). Retrospectively, these children indicated that the period between 10-13 years was crucial for their gender identity (Steensma et al., 2013). A combination of hormonal, genetic and environmental factors appears to influence gender development, but these are difficult – if not impossible – to disentangle. Gender identity is for obvious reasons impossible to study in animals, and experimental studies are unethical in humans. The best alternative is to study the relationship of hormones on gender development in a normal population via a longitudinal study from before birth to adolescence. This is what I will do in the last part of the thesis.

The second main research question in this thesis is:

What is the influence of prenatal and pubertal sex hormones on gender development from childhood to adolescence?



3 RESEARCH GROUPS, 2 UNIQUE DATASETS

Three research groups joined forces to investigate the two research questions: Clinical and Developmental Neuropsychology (University of Groningen), Behavioural Biology (University of Groningen), and the Center of Expertise on Gender Dysphoria (Medical Psychology, VU University Medical Centre Amsterdam). Each group has its own expertise and a different perspective on the sexual differentiation of brain and behaviour, and this interdisciplinary combination of research fields makes this project very interesting to me.

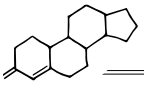
Based on a standing cooperation since 2006 with the largest gender identity clinic in the Netherlands (VUMC, Cohen-Kettenis, Kreukels and colleagues) we had the unique opportunity to use their rare longitudinal dataset of prenatal hormone concentrations and gender development from a typically developing population, and their functional brain imaging data of persons diagnosed with Gender Dysphoria before and during testosterone treatment.

DATASET 1: PRENATAL HORMONE MEASUREMENTS OF ADOLESCENTS

In the year 2000, 178 healthy pregnant women underwent an amniocentesis for prenatal diagnostic screening in the 14-18th week of pregnancy. This is exactly within the first sensitive period in which sex hormones influence brain differentiation (Knickmeyer and Baron-Cohen, 2006). Prenatal sex hormone concentrations were measured in the amniotic fluid, including testosterone, estradiol and progesterone (Van de Beek et al., 2004). The children born from these pregnancies were followed since then. Play behaviour was assessed when they were 1, 2.5 and 6 years old, to determine the effect of sex hormones on gender development. At 1 year of age, the relationship between prenatal sex hormones and toy preference was investigated (Van de Beek et al., 2009). At 6 years of age, the relationship between prenatal testosterone and lateralization of language (dichotic listening) and hand preference was investigated (Lust et al., 2011, 2010). We initiated a follow-up study when the children were 15 years old, which offered us the possibility to test – for the first time – the effect of both prenatal and pubertal hormone levels on brain lateralization and gender development. I visited 60 of these children, and assessed their pubertal sex hormone levels in saliva, their brain lateralization during 3 cognitive tasks and their gender development. It is truly unique to have access to prenatal hormone levels, especially in children that were followed for such a long time.

DATASET 2: TESTOSTERONE TREATMENT IN TRANSBOYS

Gender Dysphoria is characterized by the feeling of incongruence between experienced gender and physical appearance. Persons diagnosed with Gender Dysphoria experience distress resulting from a strong mismatch between their gender identity and their sex assigned at birth (DSM-5, APA 2013). Prevalence is now estimated to 1:3800 for men (trans women) and 1:5200 for women (trans men) in the Netherlands (Wiepjes et al., 2018). There is worldwide an enormous increase in referrals for Gender Dysphoria (Zucker, 2017). The VUmc Center of Expertise on Gender Dysphoria is renowned for their research, diagnostic work and treatment of Gender Dysphoria. It is the first institute that applied puberty suppression shortly after the onset of puberty, and cross-sex hormones from age 16 onwards as treatment (Kreukels et al., 2011). To increase understanding of Gender Dysphoria and the possible effects of puberty suppression and cross-sex hormone treatment, many studies have been performed in this center. Over the past years, brain activation of transboys (girls assigned at birth diagnosed with Gender Dysphoria) was assessed with functional Magnetic Resonance imaging (fMRI) during puberty suppression and after approximately a year of testosterone treatment. We used this data to analyze the lateralization of brain functions before and after testosterone treatment in transboys and compared this to that of control boys and control girls, providing valuable insight in the effects of testosterone manipulation on brain lateralization in humans.



OUTLINE OF THE THESIS

CHAPTER 2 is an extensive methodological chapter, in which we describe the various ways to investigate the effect of sex hormones on brain lateralization in humans and other animals. Background information on steroid hormones can be found in this chapter too. Techniques to determine structural and functional brain lateralization are discussed. Especially relevant for the following chapters is the background information on the assessment of prenatal hormones in amniotic fluid and of pubertal hormones in saliva (Chapter 3, 4 and 6), and more information on puberty suppression and cross-sex hormone therapy in persons diagnosed with Gender Dysphoria (Chapter 5).

In **CHAPTER 3**, we investigate the effect of prenatal and pubertal testosterone on lateralization with longitudinal data of adolescent boys and girls. Brain lateralization is assessed with functional Transcranial Doppler sonography during three different cognitive tasks. The Mental Rotation and Word Generation tasks were chosen as these are well established tasks to study lateralization, being in the majority of people in opposite hemispheres, and the Chimeric Faces task was included as effects of sex hormones on facial emotion processing have been found. An asset of this article is that the effect of sex hormones on lateralization of multiple tasks can be compared, as hormones could affect various brain areas differentially. Moreover, this is the first study that includes both prenatal and pubertal testosterone.

In **CHAPTER 4** the influence of prenatal and pubertal estradiol on the development of brain lateralization is investigated. The classic hypotheses on the influence of hormones on the development of brain lateralization only focus on prenatal testosterone and there is no literature on organizing effects of estradiol on the development of brain lateralization. In Chapter 3, we demonstrate that not only prenatal testosterone should be taken into account, but pubertal testosterone levels as well. In Chapter 4 we take it a step further, by investigating the effects of prenatal and pubertal estradiol on brain lateralization with the same method and in the same participants as the previous chapter.

In **CHAPTER 5** the effect of testosterone treatment on lateralization in transboys is investigated and compared to adolescent control boys and girls with naturally circulating testosterone levels. Earlier reports indicate that boys show more activity in the right amygdala than girls for the perception of emotional faces, and that testosterone affects amygdala structure and activation. Lateralization of the amygdala is assessed with functional Magnetic Resonance imaging during facial emotion recognition. This study provides valuable insight in the effects of testosterone treatment on brain lateralization in humans.

In **CHAPTER 6** the influence of sex hormones on the gender development is investigated from before birth to adolescence. The sexual differentiation of the brain is assumed to be the main causal factor of sex differences in behaviour, via an interplay between genes, sex hormones and the external environment. In this chapter we explore the effect of prenatal sex hormones on gender-typical play preferences during childhood, the effect of prenatal and pubertal sex hormones on gender

development (gender role, expression and sexual orientation) in adolescence, as well as the predictive value gender-typical play preferences in childhood on gender development in adolescence.

Finally, in the **DISCUSSION**, the findings of all these studies are synthesized in an overarching discussion, which inspired a few new analyses. First, the outcomes of the lateralization studies are analyzed in combination with each other, and next, parallels in the effect sex hormones have on brain lateralization and gender development are discussed. Furthermore, the relationship between these rather different output measures of sexual differentiation is tested. I hope to show you that that the study of the hormonal basis for human sexual differentiation is interesting and needed for a better understanding of the development of our own brain and behaviour.

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